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Glial Na_x Channels Control Lactate Signaling to Neurons for Brain [Na^+] Sensing

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SUMMARY

Sodium (Na) homeostasis is crucial for life, and Na levels in body fluids are constantly monitored in the brain. The subfornical organ (SFO) is the center of the sensing responsible for the control of salt-intake behavior, where Na_x channels are expressed in specific glial cells as the Na-level sensor. Here, we show direct interaction between Na_x channels and α subunits of Na^+/K^+-ATPase, which brings about Na-dependent activation of the metabolic state of the glial cells. The metabolic enhancement leading to extensive lactate production was observed in the SFO of wild-type mice, but not of the *Na_x*-knockout mice. Furthermore, lactate, as well as Na, stimulated the activity of GABAergic neurons in the SFO. These results suggest that the information on a physiological increase of the Na level in body fluids sensed by Na_x in glial cells is transmitted to neurons by lactate as a mediator to regulate neural activities of the SFO.

INTRODUCTION

Sodium (Na) is a major electrolyte of the extracellular fluids and the main determinant of osmolarity. Because Na homeostasis is essential to life, Na-ion concentrations in plasma and CSF are continuously monitored to maintain a physiological level of Na in body fluids. A specific Na sensor has been long hypothesized to exist in the brain for the control of Na intake (Denton et al., 1996; Weisinger et al., 1979) as well as natriuresis (Cox et al., 1987; Denton et al., 1996). The site for the sensing was postulated in the circumventricular organs (CVOs) in the periventricular region of the brain (Cox et al., 1987; Denton et al., 1996; Park et al., 1989). The CVOs, midline structures found in the brains of all vertebrates (McKinley et al., 2003), are so named because of their proximity to the ventricles of the brain. Their specialized common features are extensive vascularization, no blood-brain barrier, and atypical ependymal cells. Only three loci in the CVOs, the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and area postrema (AP), harbor neuronal cell bodies, which have efferent neural connections to many other areas of the brain. Their neurons are supposedly exposed to the chemical environment of the general circulation, unlike other neuronal perikarya in the CNS, because of the lack of a normal blood-brain barrier. Therefore, these three CVOs are termed "sensory circumventricular organs" (Johnson and Gross, 1993).

Previously, we found that Na_x, an atypical Na channel whose structure is poorly homologous to the voltage-gated Na channels (Goldin et al., 2000), is expressed in the four CVOs, the SFO, OVLT, median eminence (ME), and posterior pituitary (Watanabe et al., 2000). *Na_x*-KO mice showed marked neuronal activation in the SFO and OVLT after water deprivation compared with wild-type mice as estimated by Fos immunoreactivity (Watanabe et al., 2000). Moreover, *Na_x*-KO mice did not stop ingesting salt when dehydrated, while wild-type mice avoided salt (Hiyama et al., 2004; Watanabe et al., 2000). We subsequently demonstrated that the Na_x channel is a concentration-sensitive Na channel with a threshold value of ~150 mM for the extracellular Na ion (Hiyama et al., 2002). We further showed that salt-aversive behavior does not occur on direct infusion of a hypertonic Na solution into the cerebral ventricle in *Na_x*-KO mice in contrast to wild-type mice (Hiyama et al., 2004). The behavioral phenotype of *Na_x*-KO mice was completely recovered by a site-directed transfer of the *Na_x* gene with an adenoviral vector into the SFO (Hiyama et al., 2004). All these findings indicate that the Na_x channel is the brain's Na-level sensor (Noda, 2006) that was postulated to be

present in the CVOs involved in the regulation of water/salt intake (Andersson, 1978).

More recently, we found that Na_x channels are specifically expressed in perineuronal processes of astrocytes and ependymal cells enveloping particular neural populations in the sensory CVOs (Watanabe et al., 2006). These Na_x-positive glial cells were sensitive to an increase in the extracellular Na level, indicating that glial cells, not neurons, are the primary site of Na-level sensing. Thus, the mechanism by which the Na signal sensed by "inexcitable" glial cells is transferred to neurons has remained to be elucidated. In the present study, we show that Na_x channels stably interact with Na^+/K^+-ATPase to assist in the activation and Na^+ influx by Na_x channels stimulates anaerobic glucose metabolism in the glial cells. The resultant lactate released from the glial cells appeared to play a crucial role in the control of neuronal activities involved in the Na-intake behavior in the brain.

RESULTS

Na_x Channels Directly Interact with Na^+/K^+-ATPase

To better understand the physiological processes involving Na_x in glial cells, we screened for molecules interacting with Na_x using the yeast two-hybrid system with each of the cytoplasmic domains of mouse Na_x as bait. Among the positive clones isolated from a mouse DRG cDNA library by using the C-terminal region of Na_x (amino acid residues 1489–1681) as bait (Figure 1A, left), three clones coded for the α subunit of Na^+/K^+-ATPase. A detailed analysis revealed that all of these clones were identical and coded for the amino acid residues 596–717 of the α1 isoform, a position close to the cytoplasmic catalytic domain of the α subunit of Na^+/K^+-ATPase (Figure 1A, right; Figure 1B). The direct interaction between the C-terminal region of Na_x and the α1 subunit of Na^+/K^+-ATPase was verified by pull-down assays (see Figure S1 in the Supplemental Data available online). We further examined the interaction between the full-length mouse α1 subunit of Na^+/K^+-ATPase and full-length Na_x protein by establishing C6 cell lines expressing Na_x (C6M16; see next section). Following the immunoprecipitation of the cell lysate with a specific antibody (6H antibody) to the α1 subunit of Na^+/K^+-ATPase, a Western blot analysis with anti-Na_x-channel antibodies was performed. The 6H antibody efficiently pulled down both Na_x ($\sim$200 kDa) and the α1 subunit of Na^+/K^+-ATPase at $\sim$110 kDa (Figure 1C), demonstrating an interaction between the two proteins in living cells. Anti-HA used as a control antibody did not pull down either the Na_x channel or the α1 subunit of Na^+/K^+-ATPase (Figure 1C, Con).

Coexpression of the α1 subunit of Na^+/K^+-ATPase and Na_x channels was examined by double-fluorescent immunostaining using sections of the SFO and dissociated cells from the SFO. The α1 subunit was broadly distributed throughout the SFO, overlapping with the expression of Na_x channels (Figure 1D). The confocal microscopic analyses with isolated cells from the SFO showed that both molecules were colocalized in the plasma membrane (Figure 1E). Na_x channels were expressed in large round cells, but not in small cells with neurite-like processes: we know that the former are glial fibrillary acidic protein (GFAP)-positive glial cells including ependymal cells, and the latter are neurons (see Watanabe et al., 2006). On the other hand, the α1 subunit of Na^+/K^+-ATPase was expressed in both cell types, as expected (Figure 1E).

Because the α2 and α3 isoforms of the α subunit of Na^+/K^+-ATPase are also expressed in the nervous system (Shamraj and Lingrel, 1994), we subsequently examined the interaction of the α2 and α3 isoforms with the C-terminal region of Na_x. Experiments using the yeast two-hybrid system showed that the cytoplasmic fragment of α2 corresponding to the region of the α1 isoform isolated (amino acids 593–714, Figure 1B) also interacted with the C-terminal region of Na_x, but that of α3 (586–707, Figure 1B) did not (Figure 1F).

To know whether the three α subunit isoforms of Na^+/K^+-ATPase are coexpressed with Na_x channels in astrocytes and ependymal cells of the SFO, we performed in situ hybridization using adult mouse SFO tissue sections. The mRNAs encoding the α1 and α2 isoforms of Na^+/K^+-ATPase were expressed in the SFO with a similar pattern to the Na_x channels (Figures 1Ga–1Gc). However, signals for the α3 isoform were not detected in the SFO (Figure 1Gd).

Functional Expression of Na_x in C6 Cells

Attempts to establish stable cell lines expressing functional Na_x channels have been long unsuccessful. Therefore, we turned our attention to developing cell lines in which the expression of Na_x channel is inducible under the control of the tetracycline-responsive element (TRE). In this system, Na_x expression is induced by a transactivator, Tet-Off, which is then transfected and expressed with an adenoviral vector: it is possible to suppress the expression again in the presence of a tetracycline analog, doxycycline (Dox), in this system (Figure 2A).

C6 is a rat glioma cell line devoid of endogenous *Na_x* expression (Gautron et al., 1992). We established four such inducible cell lines from C6 cells by transfection of an expression vector for *Na_x*. Figures 2B–2D show characteristic features of one of the four cell lines, C6M16. The expression of Na_x proteins began to be detected 24 hr after the viral transfection of the Tet-Off activator (Figure 2B, lane 2). The expression was completely inhibited with Dox at a concentration of 10 ng/ml or more (Figure 2B, lane 4). Immunocytochemistry with anti-Na_x-channel antibodies confirmed these results (Figure 2C): intensive immunopositive signals were observed in the plasma membrane of the cells only when the expression of *Na_x* was induced (Figure 2Cb). Colocalization of the α1 subunit of Na^+/K^+-ATPase and Na_x channels at the subcellular level was observed in C6M16 cells by double-fluorescent immunocytochemistry (Figure S2, 145 mM).

By using an intracellular Na-ion-imaging technique, we recently demonstrated that Na_x-positive cells dissociated

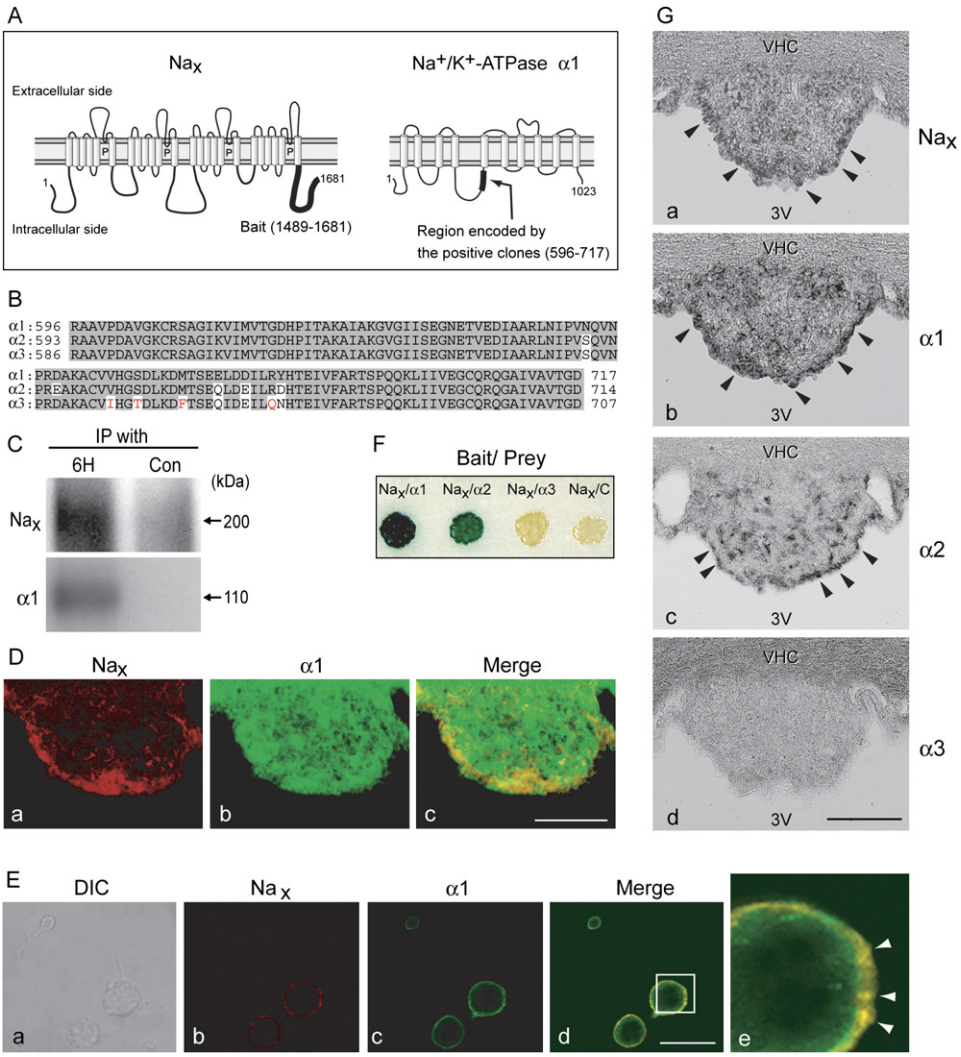

Figure 1. Interaction between Na_x Channels and α Subunits of Na^+/K^+-ATPase

(A) Schematic illustrations of Na_x channel (left) and α1 subunit of Na^+/K^+-ATPase (right). The numbers represent amino acid residues. In Na_x, the C-terminal region of the bold line represents a bait region used for the yeast two-hybrid screening. In the α1 subunit of Na^+/K^+-ATPase, the region encoded by the positive clones is drawn in bold. P, putative pore-forming regions.

(B) Alignment of amino acid sequences of the α1 (596–717), α2 (593–714), and α3 (586–707) isoforms of mouse Na^+/K^+-ATPase. Amino acids identical among them are shaded, and unique amino acids in α3 are drawn in red.

(C) Coimmunoprecipitation of Na_x channels and the α1 subunit of Na^+/K^+-ATPase. Cell lysate prepared from C6M16 cells was immunoprecipitated with a monoclonal antibody (6H) to the α1 subunit of Na^+/K^+-ATPase or anti-HA monoclonal antibody (Con) as a negative control and analyzed by Western blotting using anti-Na_x antibody (upper) or 6H (lower).

(D and E) Colocalization of Na_x channels and the α1 subunit of Na^+/K^+-ATPase on coronal tissue sections (D) and dissociated cells (E) of the mouse SFO. Immunostaining of Na_x channels (Na_x) and the α1 subunit of Na^+/K^+-ATPase (α1) is merged to show the colocalization (Merge). A higher-magnification picture of the boxed area in (Ed) is shown in (Ee). Arrowheads point to the dense colocalization of Na_x and the α1 subunit in the plasma membrane. DIC, differential interference contrast image. Scale bars: 100 μm for (D), 30 μm for (E).

(F) Filter-lift β-galactosidase assay to examine the binding of the C-terminal region of Na_x channels with the α1, α2, and α3 subunits of Na^+/K^+-ATPase. The bait is the C-terminal region of Na_x. As prey, three polypeptides derived from the third cytoplasmic domain of the α subunit of Na^+/K^+-ATPase (shown in [B]) were used (Na_x/α1–α3). As a negative control, only the GAL4-activating domain was expressed (Na_x/C).

(G) In situ hybridization with probes for Na_x channel, and α1, α2, and α3 subunits of Na^+/K^+-ATPase on coronal tissue sections of the mouse SFO. The SFO is attached to the ventral hippocampal commissure (VHC) and faces the dorsal third ventricle (3V). Positive signals were observed in ependymal cells facing the 3V (arrowheads) and cells inside the SFO with the Na_x, α1, and α2 probes, but not with the α3 probe. Scale bar: 100 μm.

from the SFO specifically show Na^+ influx in response to an increase of the extracellular Na level above the physiological level (Hiyama et al., 2002; Noda and Hiyama, 2005; Watanabe et al., 2006). In the present study, we examined whether the Na_x-expressing C6M16 cells acquired such Na sensitivity using the same method. As shown in

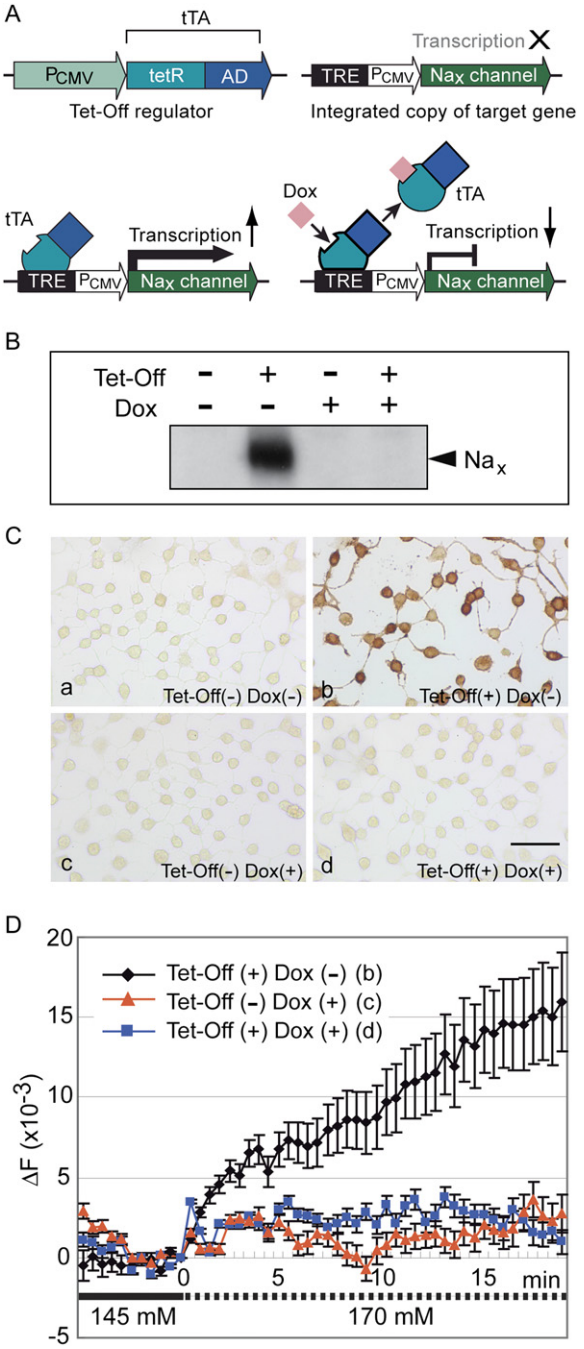

Figure 2. Functional Expression of Na_x Channels in Rat C6 Glioma Cells

(A) Schematic drawings of expression constructs and regulatory mechanisms for the expression of Na_x. The Na_x channel expression vector inducible by Tet-Off was introduced into C6 cells, and stable cell lines were established. The Tet-controlled transactivator (tTA) is a fusion of the wild-type Tet repressor protein (tetR) and the VP16 activation domain (AD) of herpes simplex virus. In the absence of doxycycline (Dox), tTA binds to the Tet-response element (TRE) and induces the expression of Na_x channels.

(B) Western blotting with anti-Na_x antibody to examine the expression of Na_x channel protein in a cell line, C6M16, with (+) or without (–) the Tet-Off regulator. Na_x protein was observed only in the induced condition. The presence of doxycycline effectively suppressed the expression of Na_x channels.

(C) Immunostaining of Na_x channels expressed in C6M16. The Tet-Off regulator induces the expression of Na_x in the absence of doxycycline (Cb). Scale bar: 50 μm.

(D) Na imaging using SBFI in C6M16 cells in a high-Na solution. The coordinate shows the fluorescent ratio (ΔF, 340/380 nm) representing the intracellular Na concentration. The fluorescent ratio at 0 min was set as zero points on the coordinate. Here, the continuous background increase by the host cell [Tet-Off(–) Dox(–)] was subtracted from respective values. The extracellular perfusion solution was changed from the 145 mM Na solution to the 170 mM Na solution at 0 min. Data are the mean and SE (n = 40 for each).

Figure 2D, C6M16 cells showed significant Na^+ influx in response to an increase in the extracellular Na level within the physiological range (from 145 mM to 170 mM) specifically under the conditions where Na_x channels are expressed. The results clearly indicated that functional Na_x channels were expressed in C6M16 cells through induction by Tet-Off. Similar results were obtained with the other three C6 lines (data not shown). Our immunocytochemical experiments indicated that the change of the extracellular Na level did not affect the colocalization of Na_x channels and the α1 subunit of Na^+/K^+-ATPase at the light microscopic level resolution (Figure S2, compare 145 mM and 170 mM), suggesting a constitutive interaction between the two.

Coupling between Na_x Channels and Na^+/K^+-ATPase Is the Basis of the Na-Dependent Metabolic Enhancement of the Cells

The close interaction of Na_x channels with the α subunits of Na^+/K^+-ATPase raised the notion that Na_x channels have a direct influence on molecular properties of the Na^+/K^+-ATPase. If it were the case, cellular energy metabolism should be affected, because cells in the CNS use ~50% of their energy resources to drive the Na^+/K^+-ATPase activity (Rosenthal and Sick, 1992). In order to explore this possibility, we measured glucose uptake activity of the C6M16 cells by using a fluorescent glucose derivative, 2-NBDG (Yoshioka et al., 1996; Yamada et al., 2000): Rb^+, which is often used for measuring activities of Na^+/K^+-ATPase directly (Serpersu and Tsong, 1984), was not suitable in this case because Rb^+ affects Na_x function (our unpublished data). C6M16 cells 36 hr after the viral transfection with or without the Tet-Off transactivator were used for the analysis (Figure 3A). The C6M16 cells did not show significant uptake of 2-NBDG in the 145 mM solution when the expression of Na_x was not induced (Con). In contrast, upon the expression of Na_x (Na_x), the cells showed substantial increase in 2-NBDG uptake in the 145 mM solution (Figure 3B, Na_x in 145 mM of C6M16). However, the enhancement was ouabain insensitive, indicating that it is independent of the Na^+/K^+-ATPase activity (compare Na_x with and without ouabain in 145 mM of C6M16 cells). Here, it should be noted that this substantial 2-NBDG uptake in C6M16 cells in the 145 mM Na solution is not observed in the native cells (see below and Figure 5).

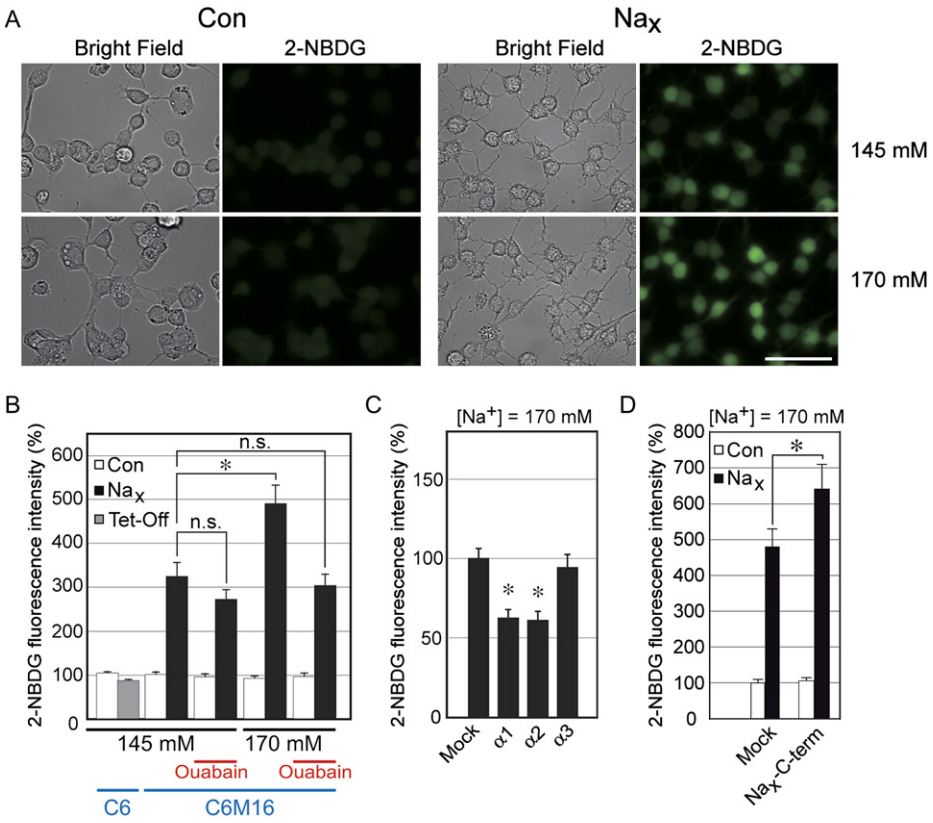

Figure 3. Interaction between Na_x and Na^+/K^+-ATPase Is the Basis for Na^+-Dependent Stimulation of Cellular Glucose Metabolism

(A) Measurements of glucose-uptake activity of C6M16 cells. Only C6M16 cells expressing Na_x (Na_x) showed uptake of a fluorescent glucose derivative, 2-NBDG. Scale bar: 50 μm.

(B) Glucose-uptake activity of C6 and C6M16 cells in various conditions. An open bar and its neighboring shaded bar on the left end are data from the native C6 glioma cells (C6) nontransfected (Con) and transfected with the Tet-Off vector (Tet-Off), respectively. The other open and closed bars are data from C6M16 cells (C6M16) nontransfected (Con) and transfected with the Tet-Off (Na_x), respectively. In some experiments, 1 mM ouabain, a specific blocker of Na^+/K^+-ATPase, was applied (Ouabain). Fluorescent intensity of Con in the 145 mM of C6M16 was set at 100%. *$p < 0.05$, Bonferroni's multiple comparison test (against Na_x in 145 mM in C6M16); n.s., not significant; data are the mean and SE ($n = 40$ for each).

(C) Glucose-uptake activity of the cells with coexpression of the Na_x-interacting region of respective α subunits of Na^+/K^+-ATPase in 170 mM Na solution. 2-NBDG uptake was significantly inhibited in Na_x-positive C6M16 cells by coexpression with the interacting regions of α1 and α2 subunits, but not of the α3 subunit, of Na^+/K^+-ATPase, compared with that in the cells transfected with the control pcDNA3.1 vector (Mock). All cells were transfected with the Tet-Off vector to induce Na_x expression and subjected to 2-NBDG uptake assays in the 170 mM Na solution. Fluorescent intensity of the Mock condition was set at 100%. *$p < 0.05$, Bonferroni's multiple comparison test (against Mock); data are the mean and SE ($n = 40$ for each).

(D) Glucose-uptake activity of the cells with coexpression of the C-terminal region of Na_x in 170 mM Na solution. C6M16 cells were transfected with the expression vector for the C-terminal region of Na_x or control pcDNA3.1 vector (Mock). Next, Na_x was not induced (Con, open bars) or induced (Na_x, closed bars) by the Tet-Off vector transfection and then used for 2-NBDG uptake assays in the 170 mM solution. The cellular fluorescent intensity of 2-NBDG was significantly enhanced in Na_x-positive C6M16 cells when transfected with the Na_x C-terminal region, compared with the cells transfected with the control vector. In Na_x-negative C6M16 cells, no such enhancement by the C-terminal of Na_x was observed. Fluorescent intensity of Con of the Mock condition was set at 100%. *$p < 0.05$, two-tailed t test (against Na_x of Mock); data are the mean and SE ($n = 40$ for each).

We then compared the cellular activities of 2-NBDG uptake in isotonic (145 mM) and hypertonic (170 mM) Na solutions. The C6M16 cells with Na_x expression (Na_x) showed ~1.6-fold greater activity for 2-NBDG uptake in the 170 mM Na solution as compared with in the 145 mM solution ($p < 0.05$), while the uptake by the C6M16 cells without Na_x expression (Con) was not increased in the 170 mM Na solution (Figure 3B, Con in 170 mM in C6M16). The increase in the uptake of 2-NBDG in Na_x-expressing cells in the 170 mM Na solution was completely inhibited by 1 mM ouabain (Figure 3B), indicating that the activity of Na^+/K^+-ATPase plays an essential role in the glucose demand induced by the elevation of the extracellular Na level.

Next, we tested the effect of overexpression of the Na_x-binding fragments (Figure 1B) of α1 and α2 subunits of Na^+/K^+-ATPase in C6M16 cells, because these fragments are expected to work as a competitor of Na^+/K^+-ATPase for binding to Na_x channels. As was expected, the transfection of an expression vector carrying the fragments of the α1 and α2 subunits significantly suppressed the metabolic response in the C6M16 cells with Na_x expression

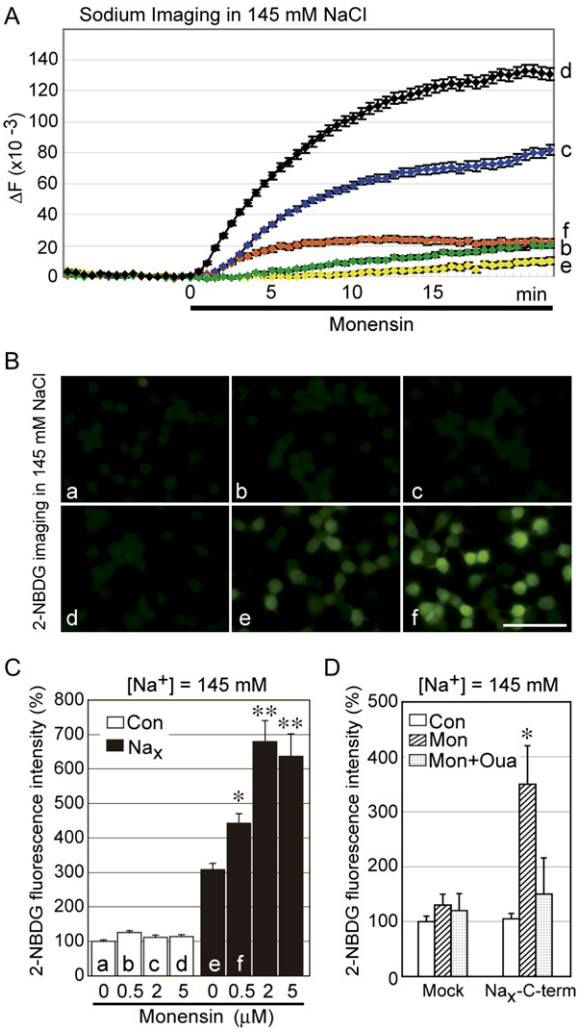

Figure 4. Activation of Glucose Uptake Requires Both Na^+ Influx and Stimulation of Na^+/K^+-ATPase by Na_x Channels through the C-Terminal Region

(A) Na^+ influx in C6M16 cells induced by monensin treatment. The coordinate gives the fluorescent ratio (ΔF, 340/380 nm) of SBFI, representing the intracellular Na concentration. The fluorescent ratio at 0 min was set as zero. The experimental conditions for (a–f) are common to (A–C): Na_x-noninduced cells (a–d), Na_x-induced cells (e and f). The 145 mM Na solution was changed to a monensin-containing 145 mM solution at 0 min; 0 μM (Ae), 0.5 μM (Ab and Af), 2 μM (Ac), or 5 μM (Ad). Background fluorescence values of Na_x-negative cells without monensin (see [Ba]) were subtracted from respective values. Data represent the mean and SE (n = 40 for each).

(B) Fluorescent images 10 min after 2-NBDG loading in 145 mM Na solutions containing monensin; 0 μM (Ba and Be), 0.5 μM (Bb and Bf), 2 μM (Bc), and 5 μM (Bd). Only Na_x-positive cells (Be and Bf) show significantly higher glucose uptake. Scale bar: 50 μm.

(C) Summary of the glucose-imaging experiments. The Na ionophore monensin markedly enhanced the glucose uptake of Na_x-expressing cells (Na_x), but not Na_x-negative cells (Con). The lower-case characters (a–f) in the bars represent respective experimental conditions common to those in (B). *$p < 0.05$, **$p < 0.01$, Bonferroni's multiple comparison test (against 0 μM of Na_x); data are the mean and SE.

(D) The role of the C-terminal region of Na_x in stimulating glucose metabolism. The C-terminal region of Na_x (Na_x-C-term) or the control pcDNA3.1 vector (Mock) was transfected to C6M16 cells, and the cells were subjected to a 2-NBDG assay with 0.5 μM monensin (Mon), or 0.5 μM monensin and 1 mM ouabain (Mon + Oua) in the 145 mM Na solution. The mean fluorescence intensity of Con (without Mon or Oua treatment) of Mock was set at 100%. Note that the expression of Na_x channels was not induced in this experiment. *$p < 0.05$, Bonferroni's multiple comparison test (against Mon of Mock); data are the mean and SE (n = 40 for each).

in the 170 mM Na solution (Figure 3C; $p < 0.05$). In contrast, overexpression of the fragment of the α3 subunit, which was negative for interaction with Na_x channel (see Figure 1F), did not suppress the metabolic activation (Figure 3C).

The C-terminal fragment of Na_x was also expected to work as a competitor for the binding of Na_x channels to Na^+/K^+-ATPase. Unexpectedly but intriguingly, overexpression of the C-terminal fragment of Na_x further enhanced the 2-NBDG uptake when it was coexpressed in Na_x-positive cells (Figure 3D, Na_x; $p < 0.05$). This suggests that the C-terminal region of Na_x is also able to support Na^+/K^+-ATPase, as well as the native Na_x. However, the expression of the C-terminal fragment of Na_x by itself (without concomitant expression of the native Na_x) exerted no effect on the 2-NBDG uptake (Figure 3D, Con). This strongly suggests that a function of the native Na_x channel (presumably Na^+-influx activity) is also essential for the upregulation of the metabolic state, in addition to the function that is substitutable with the C-terminal region of Na_x.

Na^+ Influx Is Necessary but Not Sufficient for the Metabolic Activation

To estimate the contribution of Na^+ influx itself to the metabolic activation, we tested the effect of the influx generated by a Na-specific ionophore, monensin (Harootunian et al., 1989), on the uptake of glucose. Na-imaging experiments with C6M16 cells clearly showed that monensin induced Na^+ influx in a dose-dependent manner (Figure 4A): here, background fluorescence values in the cells without monensin were subtracted from the respective values.

At a concentration of 0.5 μM, monensin triggered a small Na^+ influx into cells (Figure 4Ab), comparable to that of the C6M16 cells expressing Na_x when stimulated in the 170 mM solution (Figure 2Db). However, the application of 0.5 μM monensin to C6M16 cells without Na_x-channel expression did not enhance the 2-NBDG uptake (compare Figures 4Ba and 4Bb), and higher concentrations (2 and 5 μM) of monensin were not effective either (Figures 4Bc and 4Bd and open bars in Figure 4C). In contrast, when Na_x-expressing cells were treated with 0.5, 2, and 5 μM monensin, the 2-NBDG uptake was markedly enhanced dose-dependently (compare Figures 4Be and 4Bf and filled bars in Figure 4C). This increase was not observed in the presence of ouabain (data not shown). These results clearly indicate that the increase of the Na-ion concentration in the cell is not enough by itself to trigger the uptake of glucose (metabolic stimulation) and that the presence of Na_x-channel protein is required for the stimulation of glucose uptake by the cells.

Surprisingly and importantly, the C-terminal fragment of Na_x showed markedly enhanced 2-NBDG uptake under the condition without Na_x-channel expression with 0.5 μM monensin (Figure 4D, Mon). The increase was cancelled out by the ouabain treatment (Figure 4D, Mon + Oua). This indicates that the full-length Na_x channel can be replaced by the C-terminal fragment under the condition where Na^+ influx was secured by monensin. Taken together, it is probable that both prestimulation of Na^+/K^+-ATPase (by interaction with Na_x channels through the C-terminal region of Na_x) and Na^+ influx (through Na_x channels or monensin) are essential for the activation of the Na^+/K^+-ATPase and the cellular metabolic stimulation.

Na_x Is Involved in the Na-Dependent Enhancement of Glucose Uptake in the SFO

To examine whether the Na_x channel is indeed involved in the energy-control system in the Na_x-positive glial cells in vivo, we performed an imaging analysis of the uptake of glucose in the SFO using 2-NBDG, where specific glial cells expressing Na_x are located (Watanabe et al., 2006). The SFO from wild-type and *Na_x*-KO mice was sectioned frontally at 250 μm, incubated with 2-NBDG (200 μM) in the 145 mM or 170 mM Na solution for 1 hr, and observed with a confocal fluorescence microscope. Na-sensitive 2-NBDG uptake was obviously detected selectively in the wild-type (Figures 5Aa, 5Ab, and 5B, $p < 0.01$), but not in *Na_x*-KO (Figures 5Ac, 5Ad, and 5B) tissues: in the wild-type SFO incubated with 2-NBDG in the 170 mM solution, an intensively labeled mesh-like structure became apparent (Figure 5Ab), suggesting that fine glial processes in the SFO actively took up the fluorescent derivative of glucose. These results clearly indicate that the SFO tissue has activity to take up glucose in response to a Na-level increase, and the Na_x channel is an essential component for this mechanism. The enhancement of 2-NBDG uptake in the wild-type SFO under the high-Na condition was completely abolished by 1 mM ouabain (Figures 5Ae, 5Af, and 5B). These results indicate that the activity of Na^+/K^+-ATPase is the origin of the cellular energy demand at the tissue level in vivo.

Next, we examined the dissociated cells from the SFO to confirm that the cells showing the enhancement of 2-NBDG uptake express Na_x channels. Cells isolated from the SFO were incubated with 2-NBDG in the 145 mM or 170 mM Na solution for 20 min to estimate the glucose-uptake activity in the cells. After that, cells were fixed and stained with anti-Na_x antibody or anti-GFAP antibody. Among the wild-type cells, cellular populations that intensively took up 2-NBDG in the 170 mM solution were present, and these cells were all positive for Na_x (Figures 5C and 5D) and GFAP (Figure S3). These results clearly indicate that the Na_x channel is an essential component for the upregulation of energy demand in the SFO under the high-Na condition, as observed in the C6M16 cells.

Furthermore, the effect of monensin in the Na_x-positive C6M16 cells was reproduced in the native cells (Figure 5E). Cells dissociated from the SFO of wild-type mice showed a markedly enhanced uptake of 2-NBDG in the presence of 0.5 μM monensin, while the same stimulation of the cells from *Na_x*-KO mice induced little enhancement of the uptake.

Na_x Channels Enhance Lactate Release from the SFO Na-Dependently

Increased demand for glucose by cells means that cellular glycolysis is enhanced to yield lactate (or pyruvate). To confirm this view, we next measured the amounts of lactate and pyruvate released from the SFO as another parameter of the metabolic activity (Figure 6A). The SFO tissues removed from mice of both genotypes were incubated in a modified Ringer solution (containing 145 mM or 160 mM Na) at 37°C for 30 hr with tetrodotoxin (TTX) to reduce the influence of neuronal activities on the glial cells: Na_x is resistant to 1 μm TTX (Hiyama et al., 2002). The Ringer solutions contained 10 mM glucose and 0 mM lactate. After the incubation, lactate and pyruvate concentrations in the solutions were measured by enzyme assays using lactate oxidase and pyruvate oxidase, respectively. As was expected, the wild-type SFO showed an increase in lactate secretion by ~60% compared with the *Na_x*-KO SFO (Figure 6A, $p < 0.05$), consistent with the increase of glucose uptake (Figures 5A and 5B; WT). On the other hand, amounts of pyruvate released into the medium were 10-fold lower than those of lactate and did not differ under the two different conditions (Figure 6B). This indicates that anaerobic glycolysis was stimulated in Na_x-positive glial cells of the SFO under the high-Na condition.

Na Activates GABAergic Neurons in the SFO by a Na_x-Dependent Mechanism

We know that neurons in the SFO of *Na_x*-KO mice are hyperactivated under dehydrated conditions compared with wild-type mice (Watanabe et al., 2000), and GABAergic neurons are one of the major neuronal types surrounded by Na_x-positive glial processes in the SFO (Watanabe et al., 2006). Then we examined the neuronal activity of GABAergic neurons in the SFO under the high-Na condition. We prepared acute slices containing the SFO from *GAD-GFP* mice and *GAD-GFP/Na_x*-KO (see Watanabe et al., 2006) and recorded the firing activity of the GABAergic neurons bearing the fluorescence of GFP as marker by using patch-clamp techniques in the cell-attached mode under a fluorescence microscope. The GABAergic neurons in the SFO of both wild-type and *Na_x*-KO mice showed spontaneous firing at a similar frequency (Figures 6C and 6E: 4.25 ± 0.38 Hz for wild-type; 4.21 ± 0.35 Hz for *Na_x*-KO) under the 145 mM Na condition. After the extracellular Na concentration was raised to 160 mM, the firing frequency of the GABAergic neurons in the SFO of wild-type mice gradually increased to 7.40 ± 1.21 Hz (Figures 6C and 6E, $p < 0.05$), but that of *Na_x*-KO mice did not show a significant change (4.35 ± 0.34 Hz). In these electrophysiological experiments using tissue slices, we used 160 mM of Na, instead of the 170 mM used for C6M16 and dissociated cells. This Na concentration corresponds to

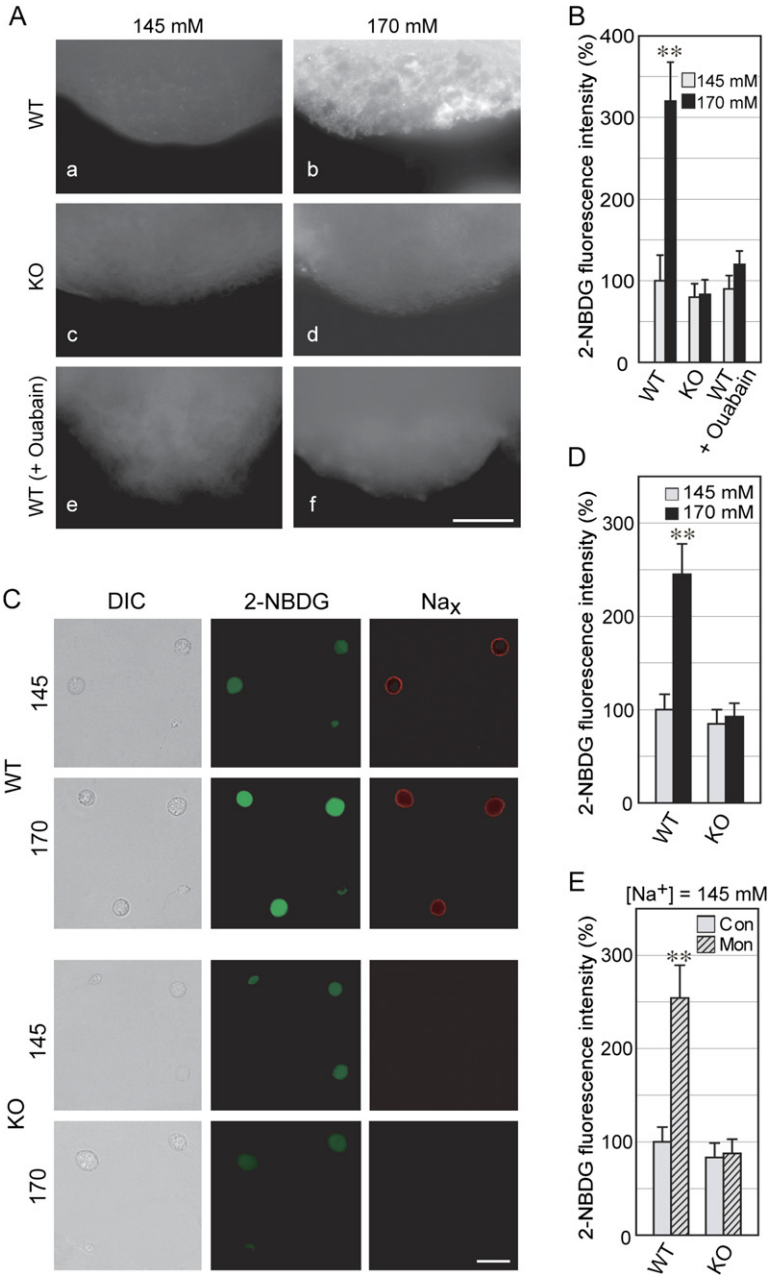

Figure 5. Na_x Channel Is Involved in Na-Sensitive Uptake of Glucose in the SFO

(A) Imaging analysis of the uptake of glucose in the SFO using a fluorescent glucose derivative. The SFO was isolated from wild-type (WT; [Aa, Ab, Ae, and Af]) and *Na_x*-KO (KO; [Ac and Ad]) mice and incubated with 2-NBDG in the 145 mM (Aa, Ac, and Ae) or 170 mM (Ab, Ad, and Af) Na solution. In some experiments, the extracellular solutions contained 1 mM ouabain (Ae and Af). The tissues did not show any significant autofluorescence before incubation with 2-NBDG (not shown). Scale bar: 50 μm.

(B) Summary of glucose-uptake activity of the SFO. Fluorescence intensities of the tissues were quantified by imaging software, and the mean and SE were obtained. The mean fluorescence intensity of the wild-type in the 145 mM Na solution was set at 100%. **$p < 0.01$, two-tailed t test (against 145 mM of WT); data are the mean and SE ($n = 5$ for each).

(C) Imaging analysis of glucose uptake in dissociated SFO cells. Cells dissociated from the SFO isolated from wild-type (WT) and *Na_x*-KO (KO) mice were subjected to imaging analysis of glucose uptake using 2-NBDG in the 145 mM or 170 mM Na solution. After the imaging, cells were stained with anti-Na_x-channel antibody (Na_x). DIC, differential interference contrast image. Scale bar: 25 μm.

(D) Summary of glucose-uptake activity of the dissociated SFO cells. The mean fluorescence intensity of wild-type (WT) cells in the 145 mM solution was set at 100%. **$p < 0.01$, two-tailed t test (against 145 mM of WT); data are the mean and SE ($n = 20$ for each).

(E) Effect of monensin on glucose uptake in dissociated SFO cells. Cells were dissociated from the SFO of wild-type (WT) and *Na_x*-KO (KO) mice. Cells were treated with 0.5 μM monensin (Mon) in the 145 mM Na solution. The mean fluorescence intensity of Con (without Mon treatment) of WT was set at 100%. **$p < 0.01$, two-tailed t test (against Con of WT); data are the mean and SE ($n = 20$ for each).

mild conditions in the physiological range of the increase, because the Na concentration in the plasma increases by 5%–10% during dehydration (Wakerley et al., 1978; Nose et al., 1992).

In addition, we examined the contribution of Na-dependent transporters in glial cells to this activation of the GABAergic neurons using inhibitors for them. Na^+ influx to glial cells reduces the ionic gradients of Na across the plasma membrane and presumably attenuates the activity of Na-dependent glutamate transporters and GABA transporters in glial cells. In the present case, however, inhibitors for these transporters did not have any significant effects on the increase in the firing rate of the GABAergic neurons in the SFO, suggesting that these transporters are not involved in this process (see Supplemental Data; Figure S4). The possibility that the effects of high Na on the firing rate were mediated by some glutamatergic neurons innervating onto the GABAergic neurons in the SFO is also very low, because antagonists of glutamate receptors did not show any effects in our electrophysiological experiments (Figure S5). Moreover, the possibility that the Na-dependent regulation of neuronal activity is mediated by the purinergic signaling process from glia to neurons with ATP (Fields and Burnstock, 2006) was ruled out by our experiments using specific inhibitors (see Figure S6).

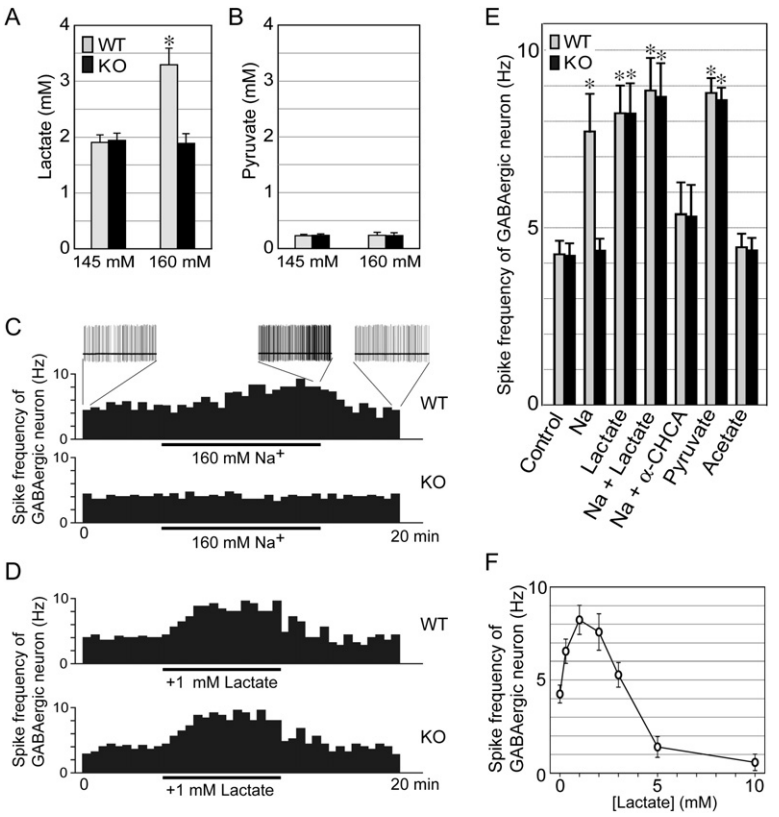

Figure 6. Lactate Activates Inhibitory Neurons in the SFO

(A and B) Release of lactate (A) and pyruvate (B) from the SFO tissue into the incubation medium. Modified Ringer solutions, in which the SFO tissues from wild-type (WT) or Na_x-KO (KO) mice were incubated for 24 hr at 37°C, were subjected to measurements by the enzyme assay. The normal modified Ringer solution and high-Na modified Ringer solution contained 145 mM and 160 mM Na, respectively. *p < 0.05, two-tailed t test (against WT of 145 mM). Data are the mean and SE (n = 10 for each).

(C and D) Control of spike frequency of GABAergic neurons in the SFO by Na and lactate. The SFO tissues from wild-type (WT) and Na_x-KO (KO) mice were treated with the high-Na modified Ringer solution (C) or 1 mM lactate in the normal modified Ringer solution (D). Na_x is indispensable for Na-dependent activation of the GABAergic neurons in the SFO, but lactate activates them independent of Na_x.

(E) Summary of the electrophysiological experiments with the SFO of wild-type (WT) and Na_x-KO (KO) mice. Means of spike frequency of the GABAergic neurons during perfusion of various kinds of solutions are shown. Na, 160 mM Na solution; Lactate, 1 mM lactate; α-CHCA, 5 mM α-cyano-4-hydroxycinnamic acid (an inhibitor of monocarboxylate transporters); Pyruvate, 1 mM pyruvate; Acetate, 1 mM acetate; *p < 0.05, Bonferroni's multiple comparison test (against WT of control). Data are the mean and SE (n = 8 for each).

(F) Concentration dependency of lactate's effect on the spike frequency of GABAergic neurons. The SFO tissues of wild-type mice were used for this experiment. Data are the mean and SE (n = 5 for each).

The Na-Dependent Neural Activation by Glial Cells Is Mediated by Lactate through Monocarboxylate Transporters

Because metabolic activation leads to the release of lactate from Na_x-positive glial cells, we next checked the possibility that lactate mediates the signal from the glial cells to GABAergic neurons to control the SFO activity. When lactate was added at 1 mM to the perfusate, the firing frequency of GABAergic neurons in the SFO of both wild-type and Na_x-KO mice increased (8.23 ± 0.78 Hz for wild-type; 8.22 ± 0.85 Hz for Na_x-KO; $p < 0.05$) (Figures 6D and 6E, Lactate). Furthermore, when 1 mM lactate was added under the high-Na condition, no additive effect on the neuronal activity was observed (8.91 ± 0.81 Hz for wild-type; 8.65 ± 0.85 Hz for Na_x-KO) (Figure 6E, Na + Lactate). These results strongly indicate that lactate and Na share a common pathway in the stimulation of GABAergic neurons in the SFO. Lactate was most effective at ~1 mM in promoting the firing rate, and at higher concentrations, the firing was rather suppressed (Figure 6F).

Lactate is known to be transported to the cytoplasmic side by monocarboxylate transporters (MCTs) and fuels the oxidative metabolism in neurons (Gladden 2004). So, we examined the effects of α-cyano-4-hydroxycinnamic acid (α-CHCA), an inhibitor of MCTs. The neural activation induced by the Na-level increase was inhibited by 5 mM α-CHCA (5.41 ± 0.92 Hz for wild-type; 5.30 ± 0.92 Hz for Na_x-KO) (Figure 6E, Na + α-CHCA). These results clearly indicate that the Na-dependent stimulation of GABAergic neurons is largely mediated by MCTs.

We also examined the effect of the other metabolic monocarboxylates, pyruvate and acetate, both known to be transported by MCTs. When pyruvate was added at 1 mM to the perfusate, the firing frequency of GABAergic neurons in the SFO of both wild-type and Na_x-KO mice similarly increased (8.80 ± 0.42 Hz for wild-type; 8.61 ± 0.35 Hz for Na_x-KO; $p < 0.05$) (Figure 6E, Pyruvate). By contrast, when acetate was added at 1 mM to the perfusate, the firing frequency was not significantly changed in either genotype (4.45 ± 0.38 Hz for wild-type; 4.36 ± 0.35 Hz for Na_x-KO) (Figure 6E, Acetate).

We further explored the activation mechanism underlying the increase in the firing rate of the GABAergic neurons. The finding that lactate and pyruvate are equally effective suggests that the GABAergic neurons are energetically stimulated. We found that Na-dependent potentiation of the firing activity of the GABAergic neurons was reduced by 0.3 mM diazoxide, an opener of the ATP-sensitive K channel (Kir6.2/K_{ATP} channel) (Figure 7A): the K_{ATP} channel closes in response to the increase of intracellular

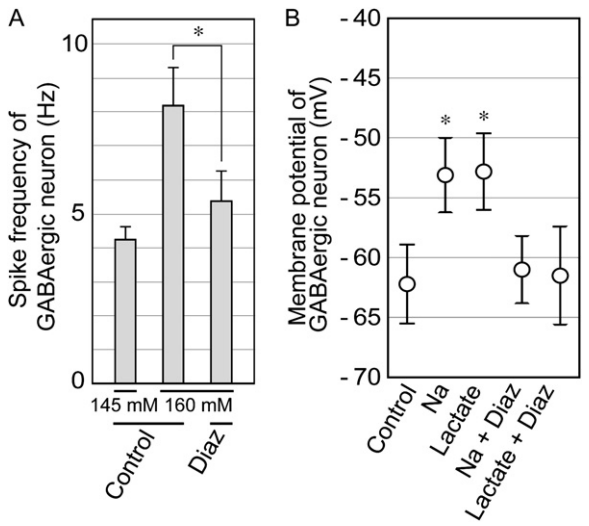

Figure 7. Putative Role of K_{ATP} Channel in Na-Dependent Stimulation of GABAergic Neurons in the SFO

Electrophysiological experiments were performed with SFO slices of wild-type mice. (A) Means of the spike frequency of GABAergic neurons during the perfusion with various kinds of solutions are shown. Diaz, 0.3 mM diazoxide, an opener of K_{ATP} channel. *$p < 0.05$, two-tailed t test (against Control of 160 mM); data are the mean and SE ($n = 5$ for each).

(B) The membrane potentials of GABAergic neurons in the presence of 1 μM TTX. Na, 160 mM Na solution; Lactate, 1 mM lactate; Diaz, 0.3 mM diazoxide; *$p < 0.05$, Bonferroni's multiple comparison test (against Control); data are the mean and SE ($n = 20$ for each).

ATP level and depolarizes the cell. So, we examined the membrane potential of the GABAergic neurons during the application of lactate or high-Na solution (Figure 7B). The membrane potential was depolarized by both lactate and Na, and the depolarization effect was expectedly reduced by 0.3 mM diazoxide. These data thus support our view that lactate serves as an energy substrate to upregulate the firing activity of the GABAergic neurons.

DISCUSSION

In the present study, we showed that there exists direct interaction between Na_x and α subunits of Na^+/K^+-ATPase, which enables the Na_x-positive glial cells to Na-dependently activate the anaerobic metabolism of glucose. Na-dependent lactate production was specifically observed in the SFO of wild-type mice, but not of *Na_x*-KO mice. Lactate was revealed to be a stimulant for the specific GABAergic neurons spontaneously firing in the SFO. Lactate released from Na_x-positive glial cells thus appears to be a neurostimulator in the SFO to control the Na-intake behavior.

Na_x Interacts with Na^+/K^+-ATPase and Na-Dependently Activates Cellular Glucose Uptake

Na^+/K^+-ATPase is composed of a catalytic α subunit and an accessory β subunit (Kaplan, 2002). There exist four isoforms of the α subunit (α1, α2, α3, and α4) with different tissue distributions (Shamraj and Lingrel, 1994). We revealed that Na_x has specific interaction with α1 and α2 isoforms of the α subunit of Na^+/K^+-ATPase (Figure 1F). The α1 and α2 isoforms are reportedly expressed in astrocytes (McGrail et al., 1991; Watts et al., 1991), consistent with the finding that the Na_x channel is expressed in glial cells in the CVOs (Watanabe et al., 2006). The expression of α1 and α2, but not α3, was detected in the SFO by in situ hybridization (Figure 1G), suggesting the functional relevancy in vivo. Of note is that the amino acid sequence corresponding to the interacting region in the α3 isoform contains significant substitutions relative to the α1 and α2 isoforms (Figure 1B). Moreover, the subunit specificity was functionally verified by coexpression experiments using the binding region of the α subunits on the glucose-uptake activity: the fragment of the α1 and α2 isoforms showed dominant-negative activity, but not that of α3 (Figure 3C). The specific interaction between Na_x and Na^+/K^+-ATPase thus appears to be indispensable for the upregulation of glucose uptake.

On the other hand, when the C-terminal fragment of Na_x was overexpressed in the Na_x-positive C6M16 cells, the Na-dependent glucose uptake was further enhanced (Figure 3D). This indicates that some of the Na^+/K^+-ATPase was free of interaction with Na_x in the cell because of a shortage of Na_x, and overexpression of the C-terminal region stimulated this population of the enzyme. For the glucose-uptake stimulation of the cell by the C-terminal region, either coexpression of the native Na_x (Figure 3D) or the application of monensin is requisite (Figure 4D). This indicates that Na^+ influx is a requirement to set Na^+/K^+-ATPase in motion, inducing demand for glucose to supply ATP to Na^+/K^+-ATPase.

It has been believed that Na^+ influx is sufficient for the activation of Na^+/K^+-ATPase by itself (Pellerin et al., 1998). However, monensin treatments alone, which actually induce extensive Na^+ influx (Figure 4A), had little effect on the Na^+/K^+-ATPase-dependent glucose-uptake stimulation in Na_x-negative cells like C6 cells (Con in Figures 4C and 4D) or the SFO primary cells (KO in Figure 5E). Only when the C-terminal fragment of Na_x (Figure 4D, Na_x-C-term) or the full-length Na_x (Figure 4C, Na_x;Figure 5E, WT) was coexpressed with Na^+/K^+-ATPase did monensin induce substantial glucose uptake. Taken all together, it is probable that preactivation (sensitization) of Na^+/K^+-ATPase occurs by the binding of Na_x and that this is essential for the Na-dependent activation and drive of Na^+/K^+-ATPase. The direct binding between Na_x and Na^+/K^+-ATPase implies a favorable situation that Na ions flowed into the cell through Na_x are directly supplied to Na^+/K^+-ATPase for being pumped out.

Recently, several molecules, including nicotinic ACh receptor, FXYD1, FXYD2, Src tyrosine kinases, and cofilin, were found to interact with Na^+/K^+-ATPase (Krivoi, et al., 2006; Feschenko et al., 2003; Zouzoulas and Blostein, 2006; Wang and Yu, 2005; Jung et al., 2002): some reportedly activate or modify the properties of Na^+/K^+-ATPase

through interaction. Here, cofilin is intriguing because it enhances Na^+/K^+-ATPase activity and binds triose-phosphate isomerase (TPI), a member of the glycolytic pathway, suggesting a direct coupling between the Na^+/K^+-ATPase and glycolytic activity (Jung et al., 2002). Of note is that clones encoding TPI were included in the positive clones in our yeast two-hybrid screening using the C-terminal region of Na_x as bait (our unpublished data).

Glial Cells Expressing Na_x Release Lactate and Regulate Neuronal Activity in a Na-Dependent Manner

It is known that activation of Na^+/K^+-ATPase leads to stimulation of anaerobic glycolysis, which leads to the production of lactate (Gladden, 2004). When the Na concentration of the incubating solution was increased to 160 mM, the SFO tissues obtained from WT mice indeed exhibited an increase in the release of lactate by ~60%, but those from the *Na_x*-KO mice showed no increase (Figure 6A). Taken together with the enhancement of glucose uptake under the high-Na condition (Figure 5A; WT, 170 mM), these results support the view that Na_x mediates the activation of Na^+/K^+-ATPase Na-dependently and stimulates anaerobic glycolysis to generate ATP to fuel it. Na_x-positive glial cells in the SFO of WT mice thus likely release lactate at higher levels, in proportion to the Na concentration in body fluids elevated under dehydrated conditions.

Our previous studies on *Na_x*-KO mice demonstrated that the Na_x channel exerts inhibitory influences on neuronal activities in the SFO, as judged from Fos immunoreactivity during dehydration (Watanabe et al., 2000). Na_x channels are expressed on perineuronal glial processes enveloping some neuronal types, including GABAergic neurons in the CVOs (Watanabe et al., 2006). Thus, the glial cells appear to regulate neural activities in the dehydrated animals to control the Na-intake behavior.

In this study, we found that there exist specific GABAergic neurons autonomously firing at ~4 Hz in the SFO: when we injected the cholera toxin B subunit for retrograde labeling to the paraventricular nucleus, a major projection site from the SFO, the labeled neurons did not overlap with the GABAergic neurons (our unpublished data), suggesting that most or all of the GABAergic neurons in the SFO are presumably not projection neurons but interneurons. The synaptic transmissions are supposedly not involved in the spontaneous firing, because antagonists of glutamate receptors exerted no significant effects on the firing (Figure S5). Consistently, some population of the neurons in the SFO reportedly showed properties of spontaneous firing even when they were isolated (Washburn et al., 2000). The presence of spontaneously firing cells at ~2 Hz in slices has been reported also in other brain regions: e.g., GABAergic stellate cells in the cerebellum and cholinergic neurons in the striatum (Kondo and Marty, 1998; Goldberg and Wilson, 2005).

Our electrophysiological data showed that the hypertonic Na solution enhanced the firing activity of the GABAergic neurons of WT but not *Na_x*-KO mice (Figure 6C; Na, Figure 6E). More importantly, lactate enhanced the activity of the GABAergic neurons of both genotypes (Figure 6D; Figure 6E, Lactate). Coapplication of high-Na and lactate did not have an additive effect on the neuronal activity (Figure 6E, Na + Lactate), indicating that the action of Na was mediated by Na_x and that the actions of Na and lactate share a common pathway to enhance the firing activity of GABAergic neurons. Because the enhancement of glucose uptake by an increase in the extracellular Na level was inhibited by α-CHCA, an inhibitor of MCTs (Figure 6E, Na + α-CHCA), MCTs, which transport monocarbonates including lactate, pyruvate, and acetate, likely mediate the effect by Na.

Pyruvate is an aerobic fuel for the tricarboxylic acid cycle and is supposedly produced from lactate by lactate dehydrogenase 1 in neurons (Gladden, 2004). Pyruvate also had a stimulating effect on the GABAergic neurons in vitro (Figure 6E, Pyruvate). However, there were no differences in the release of pyruvate between the SFO of WT and KO mice, along with no increase dependent on the Na level (Figure 6B). In contrast, acetate did not have any stimulating effect on the GABAergic neurons (Figure 6E, Acetate). Based on these findings, we concluded that lactate is the substance signaling from glial cells to neurons to activate neural activity. Consistently, it is known that lactate is preferentially metabolized in neurons rather than astrocytes, and acetate is specifically metabolized in astrocytes (Waniewski and Martin, 2004).

Lactate at 1–2 mM was most effective for the activation of GABAergic neurons, and higher concentrations of lactate actually suppressed the firing activities (Figure 6F). Because neurons have MCT2 with higher affinity (Km for lactate is ~0.7 mM), the rate of lactate uptake was readily saturated at a few mM (Hertz and Dienel, 2004). On the other hand, astrocytes have low-affinity transporters, MCT1 (Km for lactate is 3–5 mM) and MCT4 (Km is 15–30 mM); therefore, the rate of lactate uptake rises with the lactate concentration (Hertz and Dienel, 2004). The mechanism of the repression in the GABAergic neurons with the higher concentration of lactate is left for future investigation.

Lactate as a Glia-to-Neuron Signaling Substance

The conventional view that glucose oxidation fuels most activity-associated energy metabolism in neurons has recently been challenged by the astrocyte-neuron lactate shuttle hypothesis (Pellerin et al., 1998; Sibson et al., 1998; Magistretti, 2000), in which glial-produced lactate fuels neurons (Chih et al., 2001). According to the hypothesis, neural activity increases the extracellular concentration of glutamate or GABA, whose uptake by glia stimulates Na^+/K^+-ATPase and glutamine synthetase activity. This stimulates glial anaerobic glycolysis, the conversion of glucose to lactate. Glial cells release lactate, and neurons use it to fuel their activity. According to a model, ~70% of the total pyruvate/lactate that is oxidized in neurons is supplied from glial cells (Hyder et al., 2006). Moreover, it has been suggested that lactate becomes a major

substrate for neurons during prolonged stimulation, because lactate increases above the base levels (Hu and Wilson, 1997). In the present study, we revealed that lactate is the major fuel in the GABAergic neurons in the SFO (Figure 6A): the release of pyruvate was small and not regulated by the Na level (Figure 6B). For extensive firing of neurons, the activity of the neuronal Na^+/K^+-ATPase must be kept at a high level to keep the ionic gradient across the neuronal membrane. There is the possibility that continuously firing neurons like the GABAergic neurons in the SFO are more or less in an energy-deficient condition in a steady state. The lactate released from the Na_x-positive glial cells under dehydrated conditions must be specifically used as an energy source to replenish the GABAergic neurons to keep the Na^+/K^+-ATPase activity at a high level.

Here, it is possible that the effect of lactate is mediated by the K_{ATP} channel: lactate stimulates the generation of ATP through aerobic metabolism in neurons, which is responsible for closing the channel and depolarization of the neurons (Song and Routh, 2005). Our experimental data using diazoxide, an opener of the K_{ATP} channel, support the possibility that the K_{ATP} channel mediates the promotion of firing rate of the GABAergic neurons in the SFO (Figure 7A). Consistently, the deporalization effect of lactate and Na in the GABAergic neuron was reduced by diazoxide (Figure 7B). However, we cannot conclude at present that this is the sole mechanism for the stimulation of the GABAergic neurons in the SFO, because specific antagonists for K_{ATP} channel are not available. This is analogous to a recent finding that lactate is an alternate energy substrate to regulate neuronal activities of glucose-sensing neurons in the ventromedial hypothalamic nucleus (Song and Routh, 2005). However, it should be noted that lactate stimulated the GABAergic neurons in the presence of 10 mM glucose in our slice system.

Conclusion

The Na_x channel is a Na-level-sensitive Na channel that opens in response to an increase of the Na concentration in the extracellular fluid in the physiological range (Hiyama et al., 2002). Increases in Na levels in body fluids are sensed by Na_x in specific glial cells in the sensory CVOs (Watanabe et al., 2006). Among the CVOs, the SFO appears to be the center of the sensing responsible for the control of Na-intake behavior (Hiyama et al., 2004). In our last report, we showed that Na_x channels populate the cellular processes of astrocytes and ependymal cells enveloping neurons, including GABAergic neurons in the SFO (Watanabe et al., 2006). In the present study, the following cellular mechanism for the signaling from glial cells to neurons became clear (Figure 8). Na-level-dependent Na^+ influx through Na_x and direct interaction between Na_x and Na^+/K^+-ATPase are the basis for activation of Na^+/K^+-ATPase in the glial cells. Activation of Na^+/K^+-ATPase stimulates anaerobic metabolism of glucose by the glial cells, which produces lactate as the end product. There exist GABAergic neurons spontaneously firing in the SFO. Lactate released from the glial cells functions as the substance signaling to the neurons for activation. To our knowledge, this study is the first to show that glial cells take the initiative in the regulation of neural activity using lactate as a signaling substance.

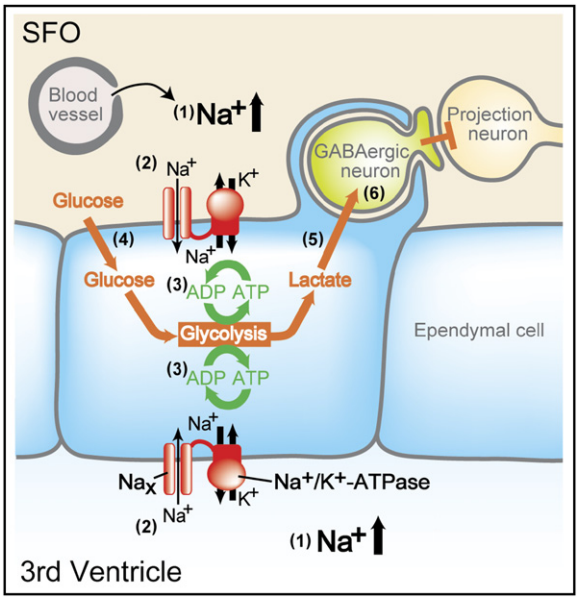

Figure 8. Schematic Overview of the Na-Level-Sensing Mechanism and Na-Dependent Regulation of Neurons in the SFO

The sensory CVOs, including the SFO, are characterized by the presence of the neuronal cell bodies and extensive networks of fenestrated capillaries which allow ingredients of plasma to release to the intercellular space. Their ventricular side is partitioned by an ependymal cell layer facing the third ventricle. Na_x channels populate perineural processes of astrocytes and ependymal cells in the SFO (Watanabe et al., 2006). When animals are dehydrated, Na concentration in plasma and CSF increases above the usual level of ~145 mM (1). When the extracellular Na concentration exceeds ~150 mM, Na_x channels open, and the intracellular Na concentration in these glial cells is increased. This leads to activation of Na^+/K^+-ATPase in these cells (2). Activated Na^+/K^+-ATPase consumes ATP higher than the usual level to pump out Na ions (3). To fuel Na^+/K^+-ATPase with ATP, the glial cells enhance the glucose uptake to stimulate the anaerobic glycolysis (4). Lactate, the end product of the anaerobic glycolysis, is released from the glial cells and supplied to neurons, including GABAergic neurons, through the processes enveloping them (5). Lactate stimulates the activity of the GABAergic neurons through production of ATP, which presumably regulate hypothetic neurons involved in the control of salt-intake behavior (6). In dehydrated KO mice, the Na-dependent stimulation of glycolysis is impaired and the activity of the GABAergic neurons is not promoted. This figure shows the case of ependymal cells; however, the same scheme would be applicable for Na_x-positive astrocytes.

EXPERIMENTAL PROCEDURES

Experimental Animals

All the experiments with animals were carried out according to the guidelines of the National Institute for Basic Biology (Okazaki, Japan).

Male wild-type (C57BL/6J, Clea Japan, Tokyo, Japan), homozygous Na_x-gene knockout (Na_x-KO), heterozygous *GAD67-GFP* (*Δneo*) knock-in (*GAD-GFP*), or *GAD-GFP* on the Na_x-KO genetic background (*GAD-GFP*/Na_x-KO) mice (Watanabe et al., 2006) were used.

For the detailed procedures and the other methods, see the Supplemental Data.

Supplemental Data

The Supplemental Data for this article, including Experimental Procedures, Supplemental Results, Supplemental References, and Supplemental Figures, can be found online at http://www.neuron.org/cgi/content/full/54/1/59/DC1/.

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